Lymphatic dysfunction and associated systemic inflammation in lymphedema, assessed by NIRF-LI imaging and plasma cytokine/chemokine analysis

Anna Vang

Follow this and additional works at: https://digitalcommons.library.tmc.edu/utgsbs_dissertations

Part of the Medical Immunology Commons, and the Quality Improvement Commons

Recommended Citation
Vang, Anna, "Lymphatic dysfunction and associated systemic inflammation in lymphedema, assessed by NIRF-LI imaging and plasma cytokine/chemokine analysis" (2023). The University of Texas MD Anderson Cancer Center UTHealth Graduate School of Biomedical Sciences Dissertations and Theses (Open Access). 1274.
https://digitalcommons.library.tmc.edu/utgsbs_dissertations/1274
Lymphatic dysfunction and associated systemic inflammation in lymphedema, assessed by NIRF-LI imaging and plasma cytokine/chemokine analysis

by

Anna Vang, B.S.

APPROVED:

Melissa B. Aldrich, MBA, Ph.D.
Advisory Professor

John C. Rasmussen, Ph.D.

Michael J. Galko, Ph.D.

Gheath Al-Atrash, Ph.D.

Pamela L. Wenzel, Ph.D.

APPROVED:

Dean, The University of Texas
MD Anderson Cancer Center UTHealth Graduate School of Biomedical Sciences
Lymphatic dysfunction and associated systemic inflammation in lymphedema, assessed by NIRF-LI imaging and plasma cytokine/chemokine analysis

A
Thesis
Presented to the Faculty of
The University of Texas
MD Anderson Cancer Center UTHealth
Graduate School of Biomedical Sciences
in Partial Fulfillment
of the Requirements
for the Degree of
Master of Science

by
Anna Vang, B.S.
Houston, Texas

May 2023
Acknowledgements

First and foremost, I would like to thank my mentor and advisor, Dr. Melissa B. Aldrich. Thank you for your guidance and support over the past 2.5 years. For having faith in my knowledge and providing words of encouragement when I needed it most.

To my advisory committee, Dr. John C. Rasmussen, Dr. Michael J. Galko, Dr. Gheath Al-Atrash, Dr. Rick A. Wetsel, and Dr. Pamela Wenzel – Thank you for providing me with the feedback needed to push my projects forward. For creating a safe space for me to brainstorm and ask questions.

To Dr. Simona F. Shaitelman and Dr. Mark V. Shaverien – the completion of the LVB and LSI studies would not be possible without you. Thank you for always being available to answer questions and providing guidance. It was an honor to have had the chance to work/collaborate with both of you.

To Dr. Chan Wenyaw – Thank you for your guidance and advice when I needed help with statistics. I could always count on your help with your quick and detailed email responses.

Lastly, I would like to thank my family and close friends, for the continuous love and support from the very beginning of my educational career. For never losing faith in me even when I did not have it myself. I would never have made it this far without your trust.
Abstract

Lymphatic dysfunction and associated systemic inflammation in lymphedema, assessed by NIRF-LI imaging and plasma cytokine/chemokine analysis

Anna Vang, B.S.

Advisory Professor: Melissa B. Aldrich, M.B.A., Ph.D.

Breast cancer-related lymphedema (BCRL) manifests as swelling of the upper extremities and trunk as a result of lymphatic fluid buildup due to radiation therapy (RT), surgical lymph node removal, or chemotherapy. As there is currently no cure, BCRL treatment aims to improve quality of life (QOL). First-line treatment involves specialized massage therapy and the use of compression garments. Second-line treatments include reparative lymphatic microsurgeries such as lymphovenous bypass (LVB) and/or vascularized lymph node transplant (VLNT). There is a need for better understanding of the etiology of BCRL and lymphatic microsurgery outcomes. Blood specimens and near-infrared fluorescent lymphatic imaging (NIRF-LI) data from two different clinical studies were used. In the first study, a total of 67 breast cancer patients were longitudinally assessed for the development of BCRL (≥5% arm swelling) after RT. A total of 314 blood specimens were collected at four different time points (preoperatively, postoperatively, and at 6- and 12-months post-RT). Fourteen plasma cytokine/chemokine levels were assessed at each time point using a MILLIPLEX MAP human cytokine/chemokine magnetic bead panel. Plasma cytokine/chemokine levels in patients with ≥5% perometric arm swelling at 12
months post-RT were compared to those with ≤5% perometric arm swelling. GraphPad/Prism 9 non-parametric Mann-Whitney test analysis was used to determine the significance of each cytokine/chemokine. In the second study, lymphatic anatomy/function metric scores and plasma cytokine/chemokine levels in 15 established BCRL patients before and at pre-, six-, and 12 months after LVB/VLNT were measured and analyzed using Image J, Excel and GraphPad/Prism 9 paired Wilcoxon analysis. In the first study, plasma cytokines/chemokines G-CSF, GM-CSF, IFN-2α, IL-10, IL-12p40, IL-15, IL-17A, IL-1β, IL-2, IL-3, IL-6, and MIP-1β were significantly elevated at pre-ALND in those with ≥5% arm swelling at 12 months post-RT compared to those who did not. Subjects only displaying dermal backflow at 12 months post-RT had elevated MIP-1β and IL-6 plasma levels at baseline. In the second study, no plasma cytokine/chemokine levels were found to be significantly different between pre- and post LVB/VLNT. Metric scores at pre-, six- and 12 months post-LVB/VLNT also displayed no significant differences. These findings suggest that plasma cytokine/chemokines could be used as biomarkers for early LE detection and determination of those at highest risk of BCRL development. Lymphatic anatomy/function metric scores and plasma cytokines/chemokines did not differ significantly pre- and post-LVB/VLNT, suggesting that these reparative microsurgeries may resolve BCRL slowly, or only in a small subset of subjects. In conclusion, these studies show that 1) BCRL is a systemic, persistently inflammatory, disease, 2) NIRF-LI is a better tool for BCRL surveillance than perometric arm volume measurement and 3) reparative microsurgeries for BCRL need further study.
Table of Contents

Approval page..............................................................................................................i
Title Page..................................................................................................................ii
Acknowledgements....................................................................................................iii
Abstract.....................................................................................................................iv
Table of Contents.......................................................................................................vi
List of Tables...............................................................................................................x
List of Figures.............................................................................................................x

Chapter 1: Introduction ..............................................................................................1
  1.1 The lymphatic system............................................................................................1
  1.2 Lymphedema/ BCRL.............................................................................................2

Chapter 2: Plasma cytokines/chemokines as predictive biomarkers for lymphedema
in breast cancer patients ...........................................................................................4
  2.1 Introduction..........................................................................................................5
  2.2 Methods...............................................................................................................8
2.2.1 Study Subjects and Design.............................................................8
2.2.2 Near-Infrared Fluorescent Lymphatic Imaging (NIRF-LI).............13
2.2.3 Perometric (RVC) Arm Volume Measurement/Clinical Diagnosis of LE........................................................................................................14
2.2.4 Extra Vascular Dye (EVD) or Dermal Backflow ......................14
2.2.5 Blood Plasma Isolation.................................................................15
2.2.6 MILLIPLEX Map Human Cytokine/Chemokine Magnetic Bead Panel........................................................................................................15
2.2.7 Statistical Analysis......................................................................16
2.3 Results............................................................................................16

2.3.1 Pre-ALND Cytokine/Chemokine Levels in Patients Who Developed BCRL 12-Months Post-RT Were Elevated..............................16

2.3.2 Several Plasma Cytokine/Chemokine Levels Were Elevated at 12 Months after RT in Those with Clinical BCRL .........................18

2.3.3 Subjects Displaying Dermal Backflow One Year after RT Showed Elevated Cytokine/Chemokine Levels at Pre-ALND ...............21

2.3.4 Several 12-Months Post-RT Plasma Cytokine/Chemokine Levels Trended Higher in Subjects with Dermal Backflow at 12-Months Post-RT ........................................................................................................23

2.3.5 Several Cytokine/Chemokine Levels Were Elevated at Pre-ALND in Subjects with Both Dermal Backflow and Clinical BCRL/LE at 12-Months Post-RT .................................................................26
2.3.6 Several 12-Months Post-RT Cytokines/Chemokines Were Elevated in Subjects with Both Clinical BCRL and Dermal Backflow Compared to Subjects with Neither Clinical BCRL nor Dermal Backflow………………………………………………………………………………………..29

2.3.7 Pearson Correlation Coefficients Comparing %RVC and %EVD to Cytokine/Chemokine Levels Exhibited Negligible to Moderate Relevance……………………………………………………………….31

2.4 Discussion…………………………………………………………………………32

2.5 Conclusion…………………………………………………………………………35

Chapter 3: The efficacy of lymphatic microsurgeries in breast cancer-related lymphedema (BCRL) patients……………………………………………………………………36

3.1 Introduction……………………………………………………………………36

3.2 Methods………………………………………………………………………..38

3.2.1 Study Subject………………………………………………………………38

3.2.2 Perometric (RVC) arm volume measurement…………………………40

3.2.3 Near infrared fluorescent lymphatic imaging (NIRFLI)………………40

3.2.4 Extra vascular dye (EVD)/ Dermal backflow…………………………..41

3.2.5 Blood specimen isolation…………………………………………………..42

3.2.6 MILLIPLEX MAP human cytokine/chemokine magnetic bead panel………………………………………………………………………………..42
3.2.7 Lymphatic and anatomy metric score……………………………42

3.2.8 Statistical analysis…………………………………………………43

3.3 Results………………………………………………………………………43

3.3.1 Subject 10 displayed a decrease in metric score from pre-surgery to six months post-surgery………………………………….43

3.3.2 Subject 10 and 11 displayed continued decrease in metric scores from pre-surgery to six-and 12 months post-surgery……………44

3.3.3 Plasma cytokine/chemokine levels comparing pre-surgery to six months post-surgery showed no significance……………………..45

3.3.4 Plasma cytokine/chemokine levels comparing pre-surgery to six months post-surgery showed no significant difference…………….47

3.4 Discussion……………………………………………………………………….50

3.5 Conclusion……………………………………………………………………..52

Bibliography………………………………………………………………………53

Vita……………………………………………………………………………………68
List of Tables

Table 1 ........................................................................................................ Page 8
Table 2 ........................................................................................................ Page 10
Table 3 ........................................................................................................ Page 31
Table 4 ........................................................................................................ Page 39

List of Figures

Figure 1 ....................................................................................................... Page 7
Figure 2 ....................................................................................................... Page 13
Figure 3 ....................................................................................................... Page 17
Figure 4 ....................................................................................................... Page 19
Figure 5 ....................................................................................................... Page 20
Figure 6 ....................................................................................................... Page 21
Figure 7 ....................................................................................................... Page 22
Figure 8 ....................................................................................................... Page 24
Figure 9 ....................................................................................................... Page 25
Figure 10 ................................................................................................. Page 27
Figure 11 ................................................................................................. Page 28
Chapter 1: Introduction

1.1 The lymphatic system

The lymphatic system consists of lymphatic vessels, lymphatic fluid, and lymphatic organs such as lymph nodes, spleen, liver, thymus, and tonsils, playing an important role in immune response. The vast network of lymphatic vessels unidirectionally filters proteins, fats, lymphocytes, and debris from small vessels in subcutaneous tissue to larger vessels deep in the muscles and adipose tissue. The lymphatic system aids in fluid homeostasis, fat absorption, and immune function [1-4].

Lymphatic vessels are made up of lymphatic capillaries, pre-collectors, and collectors, ranging from 0.5-0.8 mm in diameter [5]. Located just under the epidermis, the lymphatic capillaries are made of a single tubular layer of loose lymphatic endothelial cells (LECs) anchored to the surrounding tissues by filaments to easily allow entry of interstitial fluid. Lymphatic capillaries connect to pre-collectors, directing the flow of the lymphatic fluid through the subcutaneous tissues to larger deep vessels. The collectors are located in the subcutaneous tissues and are surrounded by smooth muscle cells that work to push the flow of lymph [6-7].

Lymph fluid flows unidirectionally throughout the lymphatic system, traveling from the distal regions of extremities, capillaries in organs, and lacteals in intestines to the cisterna chyli and then the thoracic duct, and finally into the subclavian veins to unite with the blood vasculature near the heart. Fat absorption from the liver and small
intestines is orchestrated by the lacteals within the villi of the mucosal lining for metabolism and storage [8].

The revised Starling principal [9-10] describes the physiology of flow for the primary lymphatic (capillary) system. Fluid exchange is driven by capillary pressure and interstitial osmotic pressure. Osmotic pressure increases as molecules “escape” the vascular system into interstitial spaces [8]. To reduce the capillary pressure of fluid buildup, excess molecules/protein and fluids are taken up by the lymphatic capillaries, into the lymph fluid, to be brought back into the vascular system, maintaining fluid homeostasis. Therefore, damage to any part of the lymphatic system could lead to lifelong diseases/complications such as lymphadenopathy, lymphoma, or lymphedema (LE) [9-10].

1.2 Lymphedema/Breast cancer-related lymphedema

LE is the swelling of extremities, the trunk, or the head and neck region caused by malfunctions in the lymphatic network, resulting in swollen/enlarged extremities, trunk, or head/neck, pain, increased cellulitis risk, and mental stress. The severity of LE can be divided into four different stages [12]. Stage 1 indicates abnormal lymphatic flow/early edema. Stage 2 includes edema and swelling. Stage 3 is characterized by permanent swelling and changes to the skin. Stage 4 displays scarring and skin fibrosis.

There are two different types of LE; primary LE results from abnormal formations of the lymphatic system/vessels caused by rare genetic disorders. Secondary LE, the most prominently diagnosed form of LE in developed countries, is acquired after cancer and/or RT, and is responsible for 99% of LE in adults in the US [12-13].
Breast cancer-related lymphedema (BCRL) occurs in ~40% [14-17] of breast cancer patients, resulting in chronic swelling of the upper extremities and trunk after radiation/chemotherapy and/or axillary lymph node dissection (ALND) [18-20]. Treatment options available increase quality of life (QOL) and reduce BCRL morbidity [21], but there is currently no cure [18].
Chapter 2: Plasma cytokines/chemokines as predictive biomarkers for lymphedema in breast cancer patients

This chapter is based upon:


Permission policy of MDPI and Cancers content: No special permission is required to reuse all or part of article published by MDPI, including figures and tables. For articles published under an open access Creative Common CC BY license, any part of the article may be reused without permission provided that the original article is clearly cited. Reuse of an article does not imply endorsement by the authors or MDPI.

Articles published in Cancers will be Open-Access articles distributed under the terms and conditions of the Creative Commons Attribution License (CC BY). The copyright is retained by the author(s).
2.1. Introduction

Breast cancer-related lymphedema (BCRL) is characterized by the accumulation of stagnant lymph, subdermal fat/adipose tissue, and skin fibrosis in the upper extremities and trunk after radiation, chemotherapy, and/or lymph node dissection [18,19,22]. BCRL affects approximately 40% of breast cancer survivors [15, 23-26]. As there is currently no cure [27], the management of lymphedema (LE) strives to improve quality of life (QOL). The primary treatment for LE is the use of compression to help reduce swelling and maintain arm volume reduction. In the first two weeks after LE diagnosis, patients are recommended to undergo one hour of manual lymphatic drainage (MLD) therapeutic massage daily to remove stagnant lymph, followed by immediate bandage wrapping to maintain the decreased arm volume. After maximal arm volume reduction, compression sleeves are prescribed for 24/7 wear to maintain arm volume and prevent subdermal adipose tissue buildup. If MLD fails, reparative lymphatic microsurgeries, such as vascularized lymph node transplant (VLNT) and lymphovenous bypass (LVB), can improve outcomes. VLNT serves to transplant a healthy/functional lymph node flap from an unaffected part of the body to an affected area. LVB redirects the flow of lymph by connecting affected lymphatic vessels to draining adjacent veins. These microsurgeries reduce arm swelling by 30% on average, with the continued use of compression garments [28]. The lymphatic microsurgical preventative healing approach (LYMPHA), a technique that is increasingly being adopted, creates a shunt between a lymphatic channel and a draining blood vessel at the time of ALND. One study showed that LYMPHA reduced the incidence of LE from
40% to 12.5% [29]. Treatment for LE is expensive and not fully covered by medical insurance.

BCRL’s molecular etiology is not completely known, but one study showed that stagnant lymph provides free fatty acids (FFAs) that signal subdermal adipose cells to grow and divide [30]; skin fibrosis follows, and cellulitis risk increases. Cellulitis results in trophic skin changes that can progress to sepsis if not treated or detected early, requiring hospitalization. LE also takes a toll on psychological health factors, as nearly half of breast cancer survivors have reported some level of LE-related distress and depression, anxiety, fatigue, and inability to participate in social activities [31-32].

Arm volume can be assessed by several means, including tape measurement, water displacement, or perometer measurement. Perometry determines the relative volume change (RVC) between the affected and unaffected arms. Patients with RVC scores ≥ 5% are diagnosed with LE defined by the International Society of Lymphology (ISL) [33-34]. Variable criteria for arm volume increase complicates uniform BCRL diagnosis. Breast cancer patients are not usually assessed for LE until at least 3 months after oncologic treatment, to allow for the resolution of swelling due to surgery or RT [33, 35].

“See through the skin” near-infrared fluorescence lymphatic imaging (NIRF-LI), however, has recently shown that the dermal backflow of lymph, a hallmark of BCRL, is present 8–23 months before arm swelling is evident [35]. Dermal backflow most likely results after lymphatic pumping failure, which we and others have shown can result from inflammatory cytokine actions on nitric oxide levels that can interfere with normal
nitric oxide fluctuations that drive lymphatic pumping (Figure 1) [36]. In addition to the imaging surveillance of lymphatics, biomarkers of failing lymphatic function, such as inflammatory cytokines/chemokines, could aid the early identification of breast cancer patients most at risk for developing BCRL [37-39].
Figure 1. Near-infrared fluorescence lymphatic imaging (NIRF-LI) of the right upper-proximal extremity, including the axilla, depicting (a) a healthy lymphatic structure with visible lymphatic vessels and axillary node basin, (b) dermal backflow.

We hypothesize that elevated plasma cytokine and chemokine levels precede BCRL development, and thus, could identify those at highest risk much earlier than arm volume increase. Biomarkers of BCRL risk could allow targeted surveillance and early intervention, improving patient QOL and lowering BCRL-associated medical costs.

2.2 Materials and Methods

2.2.1 Study Subjects and Design

Breast cancer patients of at least 18 years of age with no prior radiation therapy targeted to lymph nodes, who were scheduled to undergo treatment—including mastectomy or breast-conserving surgery with ALND and radiation therapy—at the University of Texas MD Anderson Cancer Center (MDACC), were recruited for the study. Breast cancer patients with additional underlying chronic illness or disease, known/suspected iodine allergy, breastfeeding, pregnancy, or an inability to keep still for a one-hour image session, were excluded from the study. The full inclusion and exclusion criteria are listed in Table 1.

Table 1. Full inclusion and exclusion criteria for recruiting study subjects

<table>
<thead>
<tr>
<th>Inclusion criteria</th>
<th>Exclusion criteria</th>
</tr>
</thead>
</table>
1) Participants must be at least 18 years of age
2) Participants must be clinically diagnosed with breast cancer
3) Patients must plan to undergo treatment with surgery and radiation therapy at MDACC
4) Clinical stage N2-N3; or clinical stage N1 with an intention to treat with axillary lymph node dissection and regional nodal radiation
5) Ambulatory and possessing all four limbs
6) No prior radiation therapy targeted to lymph nodes
7) Fluency in English or Spanish.

<table>
<thead>
<tr>
<th>1) Participants with a known or suspected allergy to iodine</th>
</tr>
</thead>
<tbody>
<tr>
<td>2) Participants who are breastfeeding, pregnant or trying to become pregnant</td>
</tr>
<tr>
<td>3) Severe underlying chronic illness or disease (other than breast cancer)</td>
</tr>
<tr>
<td>4) Participants not capable of keeping moderately still for the imaging portion of the study session (~1 hour for imaging)</td>
</tr>
</tbody>
</table>

Of the 80 study subjects who consented, 40 were excluded from our analysis—seven passed away from distant metastases before completing the study, 11 dropped out, one developed locally-regionally recurrent breast cancer, and 21 missed multiple or
analysis-relevant visits due to SARS-CoV-2. The demographics of the 40 study subjects are shown in Table 2.

Table 2. Study subject demographics.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, year, median (range)</td>
<td>48.15 (26–68)</td>
</tr>
<tr>
<td>Race, n (%)</td>
<td></td>
</tr>
<tr>
<td>Black</td>
<td>3 (7.5)</td>
</tr>
<tr>
<td>Other (Asian, American Indian/Alaska Native, multi-race)</td>
<td>5 (12.5)</td>
</tr>
<tr>
<td>White</td>
<td>32 (80)</td>
</tr>
<tr>
<td>Ethnicity, n (%)</td>
<td></td>
</tr>
<tr>
<td>Hispanic or Latino</td>
<td>6 (15)</td>
</tr>
<tr>
<td>Non-Hispanic</td>
<td>34 (85)</td>
</tr>
<tr>
<td>Sex, n (%)</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>40 (100)</td>
</tr>
<tr>
<td>Male</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Body mass index, mean (range), n (%)</td>
<td></td>
</tr>
<tr>
<td>Underweight (&lt;18.5)</td>
<td>1 (2.5)</td>
</tr>
<tr>
<td>Normal weight (18.5–24.9)</td>
<td>10 (25)</td>
</tr>
<tr>
<td>Clinical T category, n (%)</td>
<td></td>
</tr>
<tr>
<td>----------------------------</td>
<td>-------</td>
</tr>
<tr>
<td>Tx</td>
<td>1 (2.5)</td>
</tr>
<tr>
<td>T1</td>
<td>4 (10)</td>
</tr>
<tr>
<td>T2</td>
<td>15 (37.5)</td>
</tr>
<tr>
<td>T3</td>
<td>10 (25)</td>
</tr>
<tr>
<td>T4b</td>
<td>4 (10)</td>
</tr>
<tr>
<td>T4d</td>
<td>6 (15)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Clinical N category, n (%)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>N1</td>
<td>16 (40)</td>
</tr>
<tr>
<td>N2</td>
<td>4 (10)</td>
</tr>
<tr>
<td>N3a</td>
<td>7 (17.5)</td>
</tr>
<tr>
<td>N3b</td>
<td>2 (5)</td>
</tr>
<tr>
<td>N3c</td>
<td>11 (27.5)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Neoadjuvant chemotherapy, n (%)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>38 (95)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Taxanes, n (%)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>37 (92.5)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Anthracyclines, n (%)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>34 (85)</td>
</tr>
<tr>
<td>Parameter</td>
<td>Value</td>
</tr>
<tr>
<td>-------------------------------------------------------------</td>
<td>---------------</td>
</tr>
<tr>
<td>Number of lymph nodes removed at ALND, median (range)</td>
<td>23.37 (6–39)</td>
</tr>
<tr>
<td>Number of lymph nodes involved at ALND, median (range)</td>
<td>4.57 (0–36)</td>
</tr>
<tr>
<td>Lymphovascular space invasion, n (%)</td>
<td>10 (25)</td>
</tr>
<tr>
<td>Extracapsular extension, n (%)</td>
<td>13 (32.5)</td>
</tr>
<tr>
<td>Lumpectomy, n (%)</td>
<td>10 (25)</td>
</tr>
<tr>
<td>Mastectomy, n (%)</td>
<td>30 (75)</td>
</tr>
<tr>
<td>Cumulative radiation dose, Gy, median</td>
<td>49.88</td>
</tr>
<tr>
<td>Total number of fractions of radiation, median</td>
<td>26</td>
</tr>
</tbody>
</table>

Observation of each study subject was conducted over 18 months with data collection at six specified time points: pre-ALND, post-ALND, end of RT, and at 6-, 12-, and 18-months post-RT. Blood specimens and perometric scores were collected at all time points, while NIRF-LI images were collected at only five time points (Figure 2). Data from four weeks post-ALND, end of RT, and six months post-RT were excluded from this study due to insufficient numbers of subjects developing clinical LE at those time points. Data from 18 months post-RT were not used due to COVID-19 interruptions that prevented us from obtaining sufficient samples.
Figure 2. Longitudinal surveillance and data collection at pre-ALND, post-ALND, end of RT, and at 6-, 12-, and 18-months post-RT. Perometer arm measurements and blood samples were obtained at all visits. NIRF-LI image sessions were only conducted at pre-ALND, four weeks post-ALND, and at 6-, 12-, and 18-months post-RT (red asterisks).

All subjects signed their informed consent. The study was conducted under the Declaration of Helsinki and approvals from the Committees for Protection of Human Subjects/Institutional Review Boards (2016-0170 and HSC-15-1021, respectively) at both MD Anderson Cancer Center and The University of Texas Health Science Center, as well as FDA combinational Investigation New Drug application 106,345 for off-label administration and the use of ICG with NIRF-LI (NCT 02949726).

2.2.2 Near-Infrared Fluorescent Lymphatic Imaging (NIRF-LI)

The study subjects were imaged at five time points: pre-ALND, post-ALND, and at 6-, 12-, and 18-months post-RT. A total of eight intradermal injections of 0.1 cm$^3$/25 µg of indocyanine green (ICG) were made into the dorsal hand and ventral wrist areas of affected and unaffected arms at each visit, for a total dose of 200 µg. Real-time lymphatic images of the dorsal and ventral upper extremities and the axillary were obtained for each arm, for five to ten minutes per view, for a total of 30–45 min. Extremities were illuminated with 785 nm of excitation light, and the emitted fluorescence was captured by a custom, 16-bit, frame transfer, charge-coupled device camera at a field of view of 350–1900 cm$^2$ [40]. Acquired fluorescent images from each
session were processed into a stacked video file using ImageJ software (ImageJ version 1.2.4, RRID: SCR_003070) and analyzed. NIRF-LI -visible extravascular dye/dermal backflow was measured as described below.

2.2.3 Perometric (RVC) Arm Volume Measurement/Clinical Diagnosis of LE

A horizontal Perometer 400NT (Perosystem) was used to measure the RVC of each study subject at every visit. Volumetric arm measurements were calculated from the average of three perometer measurements of each arm. The formula used was RVC = (A₂U₁)/(U₂A₁) - 1, where A represents the arm volumes on the ipsilateral (affected) arm and U represents the arm volumes of the contralateral (unaffected) arm [41]. A₁ and U₁ are baseline arm volume measurements, and A₂ and U₂ are the follow-up arm volume measurements. Patients with RVC values ≥ 5% were diagnosed with clinical LE [33-34].

2.2.4 Extra Vascular Dye (EVD) or Dermal Backflow

\[
\text{BSA m}^2 = \sqrt{\left(\frac{\text{Height cm} \times \text{weight kg}}{3600}\right)} \quad (1)
\]

\[
\% \text{EVD} = \left(\left(\frac{\text{Total affected EVD cm}^2/10,000}{\text{BSAcm}^2 \times (0.09 \text{ or } 0.075)}\right)\right) \times 100 \quad (2)
\]

Using the height and weight of each subject, body surface area (BSA) was calculated (1). A single arm surface area (2), per Wallace Rule of Nines [42], was calculated as 9% of BSA for subjects with body mass index (BMI) ≤ 33, and 7.5% for subjects with BMI > 33. The percent of arm surface area displaying dermal backflow was determined by calculating the dermal backflow-affected area of the arm and
dividing by the arm surface area, by comparing the total pixels in the area of a paper rectangular grid of an area 6.0 cm² (2.0 × 3.0 cm) with the total pixels in the area of dermal backflow observed in a still NIRF-LI image, at identical distances from the NIRF-LI camera lens. If %EVD was over 1%, we considered the images positive for dermal backflow.

2.2.5 Blood Plasma Isolation

Whole blood tubes with EDTA were centrifuged at 2000× g for 20 min at four degrees Celsius. Plasma was isolated and aliquoted (170–175 µL) into microtubes before storage at −80 degrees.

2.2.6 MILLIPLEX Map Human Cytokine/Chemokine Magnetic Bead Panel

The plasma samples were analyzed using Human Cytokine/Chemokine/Growth Factor Panel A Magnetic Bead Panel 96-well plate assay purchased from Millipore Sigma (St. Louis, MI, USA) (catalog #: HCYTA-60K). A total of 14 cytokines/chemokines were run for each plasma sample: G-CSF, GM-CSF, IL-12p40, IFN-α2, IL-10, IL-15, IL-17A, IL-1β, IL-2, IL-3, IL-6, IP10, MIP-1β, TNF-α. The probable associations of these cytokines/chemokines with immune response types are as follows:

Innate: TNF-α, IFN-α2, IL-12p40, MIP-1β, IL-1β, IL-15

Adaptive: IL-2, IL-10

Both: IL-6, GM-CSF, G-CSF, IP10, IL-17A, IL-3

The plasma samples were processed using the protocol provided by the manufacturer. Plasmas from three normal healthy controls were included in the plates for verification but not used in statistical analysis.
2.2.7 Statistical Analysis

Outliers for each data set were calculated using the quartile functions in Excel to find the upper and lower bounds for each cytokine data set. Values outside those bounds were ruled out of analysis. Pearson correlation values were calculated for %RVC and %EVD with cytokine/chemokine levels. Statistical significance was determined using the Mann–Whitney unpaired nonparametric t-test and Wilcoxon paired non-parametric t-test calculated from GraphPad Prism version 9.00. *p*-values < 0.05 were deemed significant. Correlation values between 0.00 and 0.10 were considered negligible, between 0.10 and 0.39 were weak, between 0.40 and 0.69 were moderate, between 0.70 and 0.89 were strong, and between 0.90 and 1.00 were very strong [43]. Subjects who did not develop BCRL and/or dermal backflow 12 months post-RT were used as controls. For each data set, 0–2 outliers were removed if they were outside of the interquartile range.

2.3. Results

2.3.1 Pre-ALND Cytokine/Chemokine Levels in Patients Who Developed BCRL 12-Months Post-RT Were Elevated

Pre-ALND cytokine/chemokine levels for subjects diagnosed with clinical BCRL (≥5% RVC) at 12-months post-RT were compared to subjects who did not develop clinical LE at 12-months post-RT. G-CSF, GM-CSF, IFN-α2, IL-10, IL-12p40, IL-15, IL-17A, IL-1β, IL-2, IL-3, IL-6, and MIP-1β were significantly higher at pre-ALND in those who developed LE at 12-months post-RT compared to those who did not develop BCRL 12-months post-RT (Figure 3).
Figure 3. Pre-ALND plasma cytokine/chemokine levels were elevated in subjects displaying clinical BCRL/LE at 12-months post-RT. Of the 14 cytokines analyzed, only IP10 and TNF-α were not significantly elevated. ($p$-values = 0.0155, 0.0348, 0.0274, 0.0329, 0.0171, 0.0016, 0.0020, 0.0037, 0.0076, 0.0004, 0.0004, 0.0001, 0.3247, 0.2054, respectively.) * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, ns $= p > 0.05$.

2.3.2 Several Plasma Cytokine/Chemokine Levels Were Elevated at 12 Months after RT in those with clinical BCRL

Plasma IL-15, IL-3, and MIP-1β levels were found to be significantly higher at 12-months post-RT in comparison to those who did not display BCRL at 12-months post-RT (Figure 4). Other plasma cytokine/chemokine levels did not reach significance, but several trended upwards such as G-CSF with a $p$-value of 0.0593. (Figure 5).
Figure 4. Plasmas IL-15, IL-3, and MIP-1β levels at 12-months post-RT were significantly elevated in subjects who were diagnosed with clinical BCRL at 12-months post-RT compared to those who did not. (*p-values = 0.0112, 0.0027, 0.0015, respectively.) * p ≤ 0.05, ** p ≤ 0.01.
**Figure 5.** Statistically non-significant plasma cytokines/chemokines at 12 months post-RT in subjects with clinical BCRL at 12 months post-RT. (*p*-values = 0.0593, 0.2467, 0.1052, 0.5027, 0.2737, 0.5376, 0.2103, 0.3297, 0.1639, 0.3079, 0.9553, respectively.) ns = *p* > 0.05
2.3.3 Subjects Displaying Dermal Backflow One Year after RT Showed Elevated Cytokine/Chemokine Levels at Pre-ALND

Cytokine/chemokine levels at pre-ALND were analyzed, comparing those who displayed dermal backflow at 12-months post-RT to those who did not. Pre-ALND plasma IL-6 and MIP-1β were significantly higher in those who developed dermal backflow at 12-months post-RT compared to those who did not (Figure 6). Other plasma cytokine/chemokine levels did not reach significance, but IFN-α2 and TNF-α trended upwards with p-values of 0.0538 and 0.0537, respectively. (Figure 7).

**Figure 6.** Pre-ALND IL-6 and MIP-1β plasma levels were higher in subjects displaying dermal backflow at 12-months post-RT. (*p*-values = 0.0374 and 0.0071, respectively.)

* *p* ≤ 0.05, ** *p* ≤ 0.01.
**Figure 7.** Pre-ALND cytokine/chemokine levels that were statistically non-significant in subjects displaying dermal backflow at 12 months post-RT. (*p*-values = 0.3255, 0.1138, 0.0538, 0.3250, 0.1721, 0.1603, 0.5368, 0.3612, 0.3658, 0.8170, 0.7490, 0.0537, respectively.) ns = *p* > 0.05

2.3.4 Several 12-Months Post-RT Plasma Cytokine/Chemokine Levels Trended Higher in Subjects with Dermal Backflow at 12-Months Post-RT

Most cytokine/chemokine levels at 12-months post-RT were not significantly higher for those subjects with backflow at 12-months post-RT (Figure 8). Although statistically insignificant, IFN-α2, IL-12p40, IL-15, MIP-1β, and TNF-α levels trended higher in subjects who displayed dermal backflow at 12-months post-RT (Figure 9).
Figure 8. Plasma cytokine/chemokine levels at 12 months post-RT that were non-significant in subjects with dermal backflow at 12 months post-RT. (*p*-values = 0.5948, 0.6793, 0.2019, 0.7737, 0.6741, 0.6373, 0.5938, 0.7147, 0.7390, respectively.) ns = *p* > 0.05
Figure 9. IFN-α2, IL-12p40, IL-15, MIP-1β, and TNF-α levels trended higher at 12-months post-RT in subjects with dermal backflow. ($p$-values = 0.2463, 0.1071, 0.2119, 0.1230, 0.0962, respectively). ns = $p > 0.05$.

2.3.5 Several Cytokine/Chemokine Levels Were Elevated at Pre-ALND in Subjects with Both Dermal Backflow and Clinical BCRL/LE at 12-Months Post-RT

GM-CSF, IFN-α2, IL-12p40, IL-15, IL-6, TNF-α, and MIP-1β were significantly higher at the pre-ALND time point in those with both BCRL/LE and dermal backflow at 12-months post-RT in comparison to those who did not develop BCRL or dermal backflow 12-months post-RT (Figure 10). Other plasma cytokine/chemokine levels did not reach significance, but many trended upwards with $p$-values < 0.100, respectively. (Figure 11).
Figure 10. GM-CSF, IFN-α2, IL-12p40, IL-15, IL-6, TNF-α, and MIP-1β were significantly higher at pre-ALND in subjects with both clinical BCRL/LE and dermal backflow at 12-months post-RT compared to subjects with no clinical BCRL or dermal backflow. (*p-values = 0.0197, 0.0125, 0.0266, 0.0472, 0.0181, 0.0072, 0.0007, respectively.) *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.
Figure 11. Non-significant plasma cytokine/chemokine levels at pre-ALND in subjects with both clinical BCRL and dermal backflow at 12 months post-RT compared to those without clinical BCRL or dermal backflow. (*p-values* = 0.0967, 0.6925, 0.1217, 0.0841, 0.1264, 0.0942, 0.8712, respectively.) ns = *p* > 0.05
2.3.6 Several 12-Months Post-RT Cytokines/Chemokines Were Elevated in Subjects with Both Clinical BCRL and Dermal Backflow Compared to Subjects with Neither Clinical BCRL nor Dermal Backflow

IL-15, IL-3, and MIP-1β were found to be significantly higher in those who developed LE and dermal backflow at 12-months post-RT in comparison to those who did not develop LE or dermal backflow at 12-months post-RT (Figure 12). Other plasma cytokine/chemokine levels did not reach significance. (Figure 13).

![Graphs showing cytokine levels](image_url)

**Figure 12.** Plasma cytokine/chemokine levels at 12-months post-RT were elevated in those who had both clinical LE and dermal backflow at 12-months post-RT. (*p-values = 0.0449, 0.0236, 0.0041, respectively.*) *p ≤ 0.05, **p ≤ 0.01.
Figure 13. Plasma cytokine/chemokine levels at 12 months post-RT that were statistically non-significant in those with both clinical LE and dermal backflow at 12
months post-RT compared to those with neither clinical BCRL nor dermal backflow. (*p-values* = 0.1003, 0.2894, 0.1105, 0.4977, 0.0874, 0.8168, 0.3105, 0.3845, 0.4043, 0.6992, 0.2583, respectively.) ns = *p* > 0.05

2.3.7 Pearson Correlation Coefficients Comparing %RVC and %EVD to Cytokine/Chemokine Levels Exhibited Negligible to Moderate Relevance

Pearson’s coefficient was used to determine the correlation between cytokine/chemokine levels to perometric scores and dermal backflow. The correlation values for all four comparison groups yielded values between negligible and moderate correlation (Table 3). Only IL-17A and IL-1β yielded a moderate correlation when comparing cytokine levels to perometric scores.

Table 3. Pearson correlation values comparing pre-ALND and 12-months post-RT plasma cytokines/chemokine levels to %RVC and %EVD.

<table>
<thead>
<tr>
<th>Cytokine/Chemokine</th>
<th>R² for Pre-ALND pg/mL and RVC at 12-Months Post-RT</th>
<th>R² for 12-Months Post-RT pg/mL %RVC at 12-Months Post-RT</th>
<th>R² for Pre-ALND pg/mL and RVC at 12-Months Post-RT</th>
<th>R² for 12-Months Post-RT %EVD at 12-Months Post-RT</th>
</tr>
</thead>
<tbody>
<tr>
<td>G-CSF</td>
<td>0.0362</td>
<td>0.0079</td>
<td>0.0005</td>
<td>0.0066</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>0.1189 *</td>
<td>0.2475 *</td>
<td>0.0026</td>
<td>0.0344</td>
</tr>
<tr>
<td>IFN-α2</td>
<td>0.0025</td>
<td>0.3862 *</td>
<td>0.0449</td>
<td>0.0256</td>
</tr>
<tr>
<td>IL-10</td>
<td>0.0089</td>
<td>0.0107</td>
<td>0.0166</td>
<td>0.0454</td>
</tr>
<tr>
<td></td>
<td>0.0124</td>
<td>0.0818</td>
<td>0.0164</td>
<td>0.1437 *</td>
</tr>
<tr>
<td>----------</td>
<td>--------</td>
<td>--------</td>
<td>--------</td>
<td>----------</td>
</tr>
<tr>
<td>IL-12p40</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-15</td>
<td>0.0248</td>
<td>0.003</td>
<td>0.00002</td>
<td>0.1563 *</td>
</tr>
<tr>
<td>IL-17A</td>
<td>0.0384</td>
<td>0.5207 **</td>
<td>0.078</td>
<td>0.0557</td>
</tr>
<tr>
<td>IL-1β</td>
<td>0.1636 *</td>
<td>0.4829 **</td>
<td>0.0006</td>
<td>0.0296</td>
</tr>
<tr>
<td>IL-2</td>
<td>0.0965</td>
<td>0.2442 *</td>
<td>0.1477 *</td>
<td>0.0744</td>
</tr>
<tr>
<td>IL-3</td>
<td>0.0336</td>
<td>0.0712</td>
<td>0.015</td>
<td>0.0214</td>
</tr>
<tr>
<td>IL-6</td>
<td>0.0081</td>
<td>0.0889</td>
<td>0.0023</td>
<td>0.122 *</td>
</tr>
<tr>
<td>IP-10</td>
<td>0.0022</td>
<td>0.1337 *</td>
<td>0.0002</td>
<td>0.0147</td>
</tr>
<tr>
<td>MIP-1β</td>
<td>0.0442</td>
<td>0.0087</td>
<td>0.0872</td>
<td>0.006</td>
</tr>
<tr>
<td>TNF-α</td>
<td>0.0016</td>
<td>0.0348</td>
<td>0.0103</td>
<td>0.1884 *</td>
</tr>
</tbody>
</table>

Values showed * weak correlation and ** moderate correlation.

2.4. Discussion

At present, it is hard to predict who will develop BCRL. Current treatment methods only improve QOL, as there is no known cure [27]. Several studies have shown that early detection and treatment lead to better outcomes and health cost savings [35, 44].

Our recent study found that dermal backflow, detected by NIRF-LI, is an imaging biomarker that can detect lymphatic dysfunction 8–23 months before breast cancer patients are clinically diagnosed with LE by perometry [35]. In the present study, we identified plasma cytokine/chemokine biomarkers that are predictive of BCRL.
development more than a year in advance of clinically diagnosed BCRL. These findings support our hypothesis that elevated plasma cytokine and chemokine levels precede BCRL development, therefore identifying those at risk before being clinically diagnosed.

We initially started with a 38 cytokine/chemokine panel, but after three trials, we found that only 14 were relevant to our study. The elevation of these 14 cytokines/chemokines was not consistent for all comparisons and we found no relevance to innate/adaptive cytokines as TNF-α, IFN-α2, IL-12p40, MIP-1β, IL-1β, and IL-15 are involved in the innate immunity, IL-2 and IL-10 are involved in adaptive immunity, and IL-6, GM-CSF, G-CSF, IP10, IL-17A, and IL-3 are involved in both innate and adaptive immunity [45-53]. For example, IL-15, IL-3, and MIP-1β were elevated at 12-months post-RT in those with clinical LE at 12-months post-RT, while only IL-15 and MIP-1β were elevated at 12-months post-RT in those with both clinical LE and dermal backflow at 12-months post-RT. Only IL-6 and MIP-1β were elevated at pre-ALND in those displaying dermal backflow at 12-months post-RT, while no cytokines were elevated at 12-months post-RT in those displaying dermal backflow at 12-months post-RT. Despite the lack of uniformity of elevated cytokine/chemokine levels at different time points, our analysis indicates a predictive value for many of these cytokines/chemokines as biomarkers and evidence of an ongoing inflammatory presence in BCRL.

The elevation of IFN-α2, IL-12p40, IL-15, MIP-1β, and TNF-α at 12-months post-RT in subjects displaying dermal backflow at 12-months post-RT, while not statistically significant, further supports the concept that BCRL is characterized by persistent inflammation and warrants further study. MIP-1β, IL-15, and IL-1β were either trending or significantly elevated in both the clinically diagnosed BCRL group and the dermal
backflow group at both pre-ALND and 12-months post-RT. Of note, MIP-1β coerces cells to produce TNF-α, IL-1β, and IL-6, which studies have shown to dampen lymphatic pumping [36, 54-55]. TNF-α levels, although never statistically significant, were consistently elevated in those displaying dermal backflow. IL-15 strongly associates with the defense and modulation of immune cells in both innate and adaptive immunity [56], and IL-1β mediates the release of other pro-inflammatory cytokines [57-58], suggesting a vicious cycle of inflammation associated with BCRL [59].

The Pearson correlation values for the perometry readings and cytokine/chemokine levels were likely affected by the use of compression once dermal backflow was detected. Arm volumes usually decrease with the use of MLD and compression, and may not reflect the cryptic dysfunctions seen with NIRF-LI.

The key limitations of this study include the small sample size and uncertainty of compression use. Despite recruiting 80 subjects, we lost half of them due to COVID-19, death, and/or other health complications, severely reducing our sample size. A larger sample size, with a burden analysis, using odds ratios and 95% confidence intervals derived with logistic regression, could be used to produce a BCRL predictive test kit similar to those used for breast cancer susceptibility genetic screening [60]. Plasma cytokine levels could be validated in a future study using MILLIPLEX kits produced by other manufacturers and individual ELISA kits. We could not directly gauge the effects of compression use once dermal backflow and/or BCRL were detected, as we had no way to monitor subject compression use. A future study measuring cytokine/chemokine levels after monitored compression use could verify the effects on inflammation.
2.5. Conclusions

Using cytokine/chemokine MILLIPLEX assays and NIRF-Li imaging, we found elevated cytokines/chemokines at baseline/pre-ALND to be predictive of who will develop BCRL and dermal backflow more than one year later. In addition, we found evidence of an ongoing cycle of inflammation associated with BCRL one year after RT, suggesting that BCRL is a systemic disease.

Cytokine screenings could be offered to patients at highest risk, particularly Black cancer patients, as well as inflammatory breast cancer (IBC) and triple-negative breast cancer patients [19,61-62]. In cases of positive screening results, early physiotherapy and/or reparative microsurgeries, as well as anti-inflammatory dietary, exercise, and pharmaceutical interventions, could be prescribed. Future studies could determine optimal timing and duration for such therapies. In summary, our findings establish BCRL as a perpetual inflammatory disorder and suggest the use of plasma cytokine/chemokine levels to predict those at highest risk.
3.1 Introduction

Lymphatic microsurgeries are second-line treatment methods for LE with an aim to clear lymphatic fluid by re-establishing normal lymphatic function. Microlymphatic surgeries to treat LE were first introduced in 1977 by Bernard M. O’Brien [5, 63]. Since then, new microsurgical techniques such as LVB and VLNT have been established for LE treatment and have shown some success in reducing edema.

LVB or lymphoveneous anastomosis bypass surgery aims to connect a dysfunctional lymphatic vessel to a draining/recipient vein [64]. Patients in LE stages 1 or 2 who have shown little improvement with conservative treatment methods are well suited to undergo LVB, more so than those in LE stages 3 or 4 [65]. The LVB procedure begins with the use of ICG lymphography to guide and identify appropriate lymphatic channels. Isosulfan blue is then injected at the incision site for live visualization during surgical procedure. End-to-end, side-to-side, or end-to-side anastomosis can be done depending on the size of the recipient vein compared to the lymphatic vessel [5]. Patients are placed in compression garments post-operation to maintain limb volume reductions. Successful arm limb volume reduction following LVB has been reported in several studies [28, 66-69].

Vascularized lymph node transplant (VLNT) is the removal of a healthy donor lymph node flap from another part of the recipient’s body and transplantation to the
affected lymphedemous area to improve lymphatic drainage. The mechanism of VLNT is not fully understood, but one theory is that the transplanted lymph node flap induces local lymphangiogenesis between the transferred nodes and recipient site by production of vascular endothelial growth factor-C (VEGF-C) [5, 70], or takes up the surrounding lymphatic fluid, returning it back to the vascular system [5, 71]. Patients displaying dermal backflow and non-functioning lymphatics are well suited to undergo VLNT. Donor lymph node flaps for VLNT can be taken from the groin, axillary-thoracic, sub clavicular, and/or submental regions, but the most ideal donor site for VLNT is from the groin, as the donor flap can be transferred alone or continually with the abdominal flaps in autologous breast reconstruction [5, 72]. LVB and VLNT lymphatic microsurgeries have shown to be more effective than conventional treatments, with several studies showing reduction in arm swelling of approximately 44.68% [73], and little to no risk of developing cellulitis [74-75]. These microsurgeries are most effective only when there is a possibility for recovery in the earlier stages of LE. The donor site morbidity for VLNT remains at risk for further LE development, in addition to a 50% chance of arm volume increase [73, 76]. There needs to be objective assessment of the effectiveness of LE reparative microsurgeries to provide guidance for optimal results and to provide insight into any inflammatory responses that could be influencing outcomes of these microsurgeries.

We hypothesize that microsurgeries improve lymphatic anatomy and function, and we aim to provide an objective assessment of what is happening “under the skin” with NIRF-LI imaging. Plasma cytokine/chemokine levels before and after microsurgery could provide an explanation for any systemic alterations in lymphatic function.
3.2 Methods

3.2.1 Study Subject

All consented participants were/will be observed over an 18-month period with data collection of NIRF-LI images and blood specimen at pre-surgery, six, 12, and 18 months post-surgery (Figure 14, study is ongoing). Currently, there are 36 of 40 consented participants, completing 81 of 160 visits. Of the 81 completed visits, 24 resulted in no blood collection and only NIRF-LI image collection. All study subjects who fit within inclusion and exclusion criteria (Table 4) of this study were recruited from MDACCs plastic surgery department (Mark V. Schaverien, MD).

Figure 14. A longitudinal study on BCRL patients, assessing the efficacy of lymphatic microsurgeries over a span of 18 months post-surgery. NIRF-LI images, perometric arm volume, and blood specimens were obtained at all four visits (pre-surgery and six, 12, and 18 months post-surgery) as indicated by red asterisks.

Table 4. Inclusion and exclusion criteria for study recruitment. Participants have to meet all inclusion criteria to partake in study. Participants meeting any of the exclusion criteria at baseline are excluded from study.
<table>
<thead>
<tr>
<th><strong>Inclusion criteria</strong></th>
<th><strong>Exclusion criteria</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) Diagnosed with breast cancer-related lymphedema of either one or both arms</td>
<td>(1) Pregnant or breast-feeding, or trying to become pregnant</td>
</tr>
<tr>
<td>(2) At least 18 years old and capable of providing consent to participate</td>
<td>(2) Known or suspected allergy to iodine</td>
</tr>
<tr>
<td>(3) No prior LVB or VLNT surgeries</td>
<td>(3) Severe underlying chronic illness or disease (other than lymphedema)</td>
</tr>
<tr>
<td>(4) Ambulatory and possess all four limbs</td>
<td>(4) Unable to remain stationary for one hour</td>
</tr>
<tr>
<td>(5) Willing and able to comply with the study protocol requirements and all study-related visit requirements</td>
<td>No vulnerable populations will be recruited for this study.</td>
</tr>
<tr>
<td>(6) Negative urine pregnancy test within 36 hours prior to study drug administration, if of childbearing potential. Females of childbearing potential must have a negative urine pregnancy test within 36 hours prior to study drug administration and agree to use a medically accepted method of contraception for a period of one month following the study.</td>
<td></td>
</tr>
</tbody>
</table>

3.2.2 Perometric (RVC) arm volume measurement
A horizontal Perometer 400NT (Perosystem) was used to measure the arm RVC of each study subject at every visit. Changes from baseline in volumetric arm measurements, expressed as %RVC, were calculated from the average of three perometer measurements of each arm. The formula used was RVC = \((A_2U_1)/(U_2A_1) - 1\), where \(A\) represents the arm volume on the ipsilateral (affected) arm and \(U\) represents the arm volume of the contralateral (unaffected) arm [41]. \(A_1\) and \(U_1\) are baseline arm volume measurements, and \(A_2\) and \(U_2\) are the follow-up arm volume measurements. Patients with RVC values \(\geq 5\%\) were diagnosed with clinical LE [33-34].

3.2.3 Near infrared fluorescent lymphatic imaging (NIRFLI)

The study subjects were imaged at four time points: pre-surgery, six-, 12-, and 18-months post-surgery. A total of eight intradermal injections of 0.1 cm³/25 µg of indocyanine green (ICG) were made into the dorsal hand and ventral wrist areas of affected and unaffected arms at each visit, for a total dose of 200 µg. Real-time lymphatic images of the dorsal and ventral upper extremities and the axillary were obtained for each arm, for five to ten minutes per view, for a total of 30–45 min. Extremities were illuminated with 785 nm of excitation light, and the emitted fluorescence was captured by a custom, 16-bit, frame transfer, charge-coupled device camera at a field of view of 350–1900 cm² [40]. Acquired fluorescent images from each session were processed into a stacked video file using ImageJ software (ImageJ version 1.2.4, RRID: SCR_003070) and analyzed. NIRF-LI-visible extravascular dye/dermal backflow was measured as described below.
3.2.5 Extra vascular dye (EVD)/ Dermal backflow

\[
\text{BSA } m^2 = \sqrt{\frac{(\text{Height cm} \times \text{weight kg})}{3600}} \quad (1)
\]

\[
\% \text{ EVD} = \left( \frac{\text{(Total affected EVD } cm^2/10,000)}{\text{(BSA}cm^2 \times (0.09 \text{ or } 0.075)} \right) \times 100 \quad (2)
\]

Using the height and weight of each subject, body surface area (BSA) was calculated (1). A single arm surface area (2), per Wallace Rule of Nines [42], was calculated as 9% of BSA for subjects with body mass index (BMI) \( \leq 33 \), and 7.5% for subjects with BMI \( > 33 \). The percent of arm surface area displaying dermal backflow was determined by calculating the dermal backflow-affected area of the arm and dividing by the arm surface area, by comparing the total pixels in the area of a paper rectangular grid of an area 6.0 cm\(^2\) (2.0 \times 3.0 cm) with the total pixels in the area of dermal backflow observed in a still NIRF-LI image, at identical distances from the NIRF-LI camera lens. If \%EVD was over 1%, we considered the images positive for dermal backflow.

3.2.6 Blood specimen isolation

Whole blood tubes with EDTA were centrifuged at 2000\( \times \) g for 20 min at four degrees Celsius. Plasma was isolated and aliquoted (170–175 µL) into microtubes before storage at –80 degrees.
3.2.7 MILLIPLEX MAP human cytokine/chemokine magnetic bead panel

The plasma samples were analyzed using Human Cytokine/Chemokine/Growth Factor Panel A Magnetic Bead Panel 96-well plate assay purchased from Millipore Sigma (St. Louis, MI, USA) (catalog #: HCYTA-60K). A total of 14 cytokines/chemokines were run for each plasma sample: G-CSF, GM-CSF, IL-12p40, IFN-α2, IL-10, IL-15, IL-17A, IL-1β, IL-2, IL-3, IL-6, IP10, MIP-1β, and TNF-α. Plasma samples from three normal healthy controls were included in the plates for verification but not used in statistical analysis.

3.2.7 Lymphatic and anatomy metric score

The metric score, a nomogram, is used to track the changes in lymphatic function and vessel anatomy before and after microsurgery(s). It was derived from an odds ratio between 12 healthy controls and 12 LE subjects (NCT00833599). The scoring system was validated with 20 LE and 20 no-LE subjects from a different study cohort of clinical BCRL patients (NCT02949726). Based on the nomogram, subjects were given a score based on displayed dermal backflow (100 points), failure of dye migration to proximal lymph node basin (2 points), dye migration/pulsing in wrong direction (6 points), tortuosity index > 1.1 (2 points), pulsing frequency of affected ventral forearm out of range (49 points), pulsing frequency of unaffected ventral forearm out of range (4 points), pulsing frequency of affected dorsal forearm out of range (0 points), pulsing frequency of unaffected dorsal forearm out of range (4 points), pulsing frequency of
affected axilla view/upper arm out of range (4 points), and/or pulsing frequency of unaffected axilla view/upper arm out of range (2 points). If subject displayed any of these, the points, added to %EVD, made up their metric score. Metric scores were calculated at each visit to see if scores would decrease after each visit to indicate improved lymphatic function and anatomy after microsurgeries.

3.2.8 Statistical analysis

Statistical significance comparing plasma cytokine/chemokine levels was determined using Wilcoxon matched-pairs signed rank test from GraphPad Prism version 9.00. *p*-values ≤0.05 were deemed significant and *p*-values ≥0.05 were deemed insignificant.

3.3 Results

3.3.1 Subject 10 displayed a decrease in metric score at six months post-surgery

Metric scores comparing pre-surgery to six months post-surgery for the first 15 subjects displayed fairly little change except for subject 10 (Figure 15). From pre-surgery to six months post-surgery, subject 10 significantly dropped from a metric score of 284.86 to 205.86, while subjects 3, 4, 5, 6, 7, 9, 14, and 15 displayed slight increases to their metric scores from pre-surgery to six months post-surgery. Metric scores for subjects 1, 2, 8, 11, 12, and 13 showed slight decreases.
Figure 15. Metric scores for subjects one through 15 using NIRF-LI images at pre-surgery and six months post-surgery.

3.3.2 Subject 10 and 11 displayed continued decrease in metric scores from pre-surgery to six and 12 months post-surgery

Metric scores at pre-surgery, six-, and 12 months post-surgery for subjects 2, 3, 5, 7, 8, 10, and 11 were compared. These subjects are the only ones who have completed three visits so far. Subjects 10 and 11 displayed continued decrease in metric score from pre-surgery to 12 months post-surgery. Subject seven displayed a steady increase in metric score from pre-surgery to 12 months post-surgery (Figure 16).
Figure 16. Metric scores for subjects 2, 3, 5, 7, 8, 10, and 11 with NIRF-LI images at pre-surgery, six-, and 12 months post-surgery.

3.3.3 Dermal backflow of affected arm show infrequent changes

%EVD of the affected arm was calculated at each arm position (dorsal, ventral, and axilla) for subjects 2, 3, 5, 7, 8, 10, and 11 (Figure 17). Subject 3 displayed dermal backflow on both left and right arms. Subject 8 displayed a steady increase in dermal backflow on the ventral and axilla, eventually developing dermal backflow on the dorsal hand. Subject 10 displayed significant drop in %EVD 6 months post-surgery but showed increase in %EVD at 12 months post-surgery in the axilla. Overall, almost no subjects displayed consistent or significant changes in %EVD after surgery.
**Figure 17.** Dermal backflow of dorsal, ventral, and axilla of affected arm for subjects 2, 3, 5, 7, 8, 10, and 11. Affected arm is indicated on the x-axis along with subject number.
Subject 10 displayed continual decrease in dermal backflow on their affected dorsal and ventral arm, while dermal backflow of their affected axilla increased at 12 months post-surgery.

3.3.4 Plasma cytokine/chemokine levels comparing pre-surgery to six months post-surgery showed no significant difference.

Pre-surgery plasma cytokines/chemokines for the first 13 subjects displayed no significant increase or decrease at six months post-surgery (Figure 18).
Figure 18. Plasma cytokine/chemokine levels for the first 13 subjects at pre-surgery compared to six months post-surgery showed no significant difference. \((p-values = 0.5693, 0.6772, 0.3142, >0.9999, 0.2734, 0.2661, 0.4973, 0.3757, 0.5068, 0.7471, 0.1265, 0.5029, 0.3054, 0.6221, \) respectively.) \(ns = p > 0.05\)
3.4 Discussion

BCRL patients are sentenced to a lifelong battle to control arm and trunk volume increases, cellulitis bouts, depression, and pain. Current treatment methods only serve to improve QOL. Although previous studies have reported that lymphatic microsurgeries reduce arm swelling by ~ 40% [29], BCRL patients must still suffer a lifetime of compression garment use.

In this study, we found little to no change in metric scores of LE severity from pre-surgery (baseline) to a year after surgery. These findings refute our hypothesis that microsurgeries improve lymphatic anatomy and function, therefore supporting the findings of our previous study that BCRL is a persistent systemic disease.

Arm volume reduction without any improvement to the lymphatic function could be due to the removal of adipose/fat tissue during LVB/VLNT. The use of compression garments could also affect the reduction in swelling. These findings refute the purpose of these microsurgeries, as there is no recruitment of alternate vessels, growth of new vessels, or increased pumping through existing vessels visible in the NIRF-LI images so far. As shown by ICG lymphography, the presence of dermal backflow is still evident a year after surgery in the subjects we surveilled.

Cytokine MILLIPLEX plate results indicated no alterations in levels between pre-surgery and six months post-surgery, further indicating no improvement to lymphatic function. Even with perometric arm reduction, systemically, there is no immune milieu change that could affect lymphatic function as our previous work shows that BCRL is a
systemic disease [63, 77], leaving patients with a 50% chance of swelling recurrence [76-78]. Of note, a recent study by others showed that VLNT donor sites exhibited similar microscopic tissue changes (immune cell deposition, skin fibrosis, adipose buildup) to changes seen at recipient sites [79]. If “bad” donor tissue is transplanted to affected sites, minimal-to-no improvement is to be expected.

The key limitations of this study include the effects of the pandemic and the occasional failure of blood specimen collection by study coordinators. This project was originally planned to begin in 2020, but the start was delayed to 2021 due to the impact of the pandemic. Additionally, we were not able to prevent the 30% non-collection of blood specimens due to subject dehydration, same-day, or previous blood draws for other tests at MDACC, and vein sclerosis due to multiple cancer treatment-related blood draws. Even with early notification prior to appointment, blood was not collected from study subjects who came in with a port or would simply not bleed due to overused, sclerotic veins.

Future directions for this study could include comparison of baseline plasma cytokine/chemokine levels to levels at 12- and 18 months post-surgery, providing insight into the long-term systemic impact of LVB/VLNT. A study exploring lymphatic flow effects on BCRL development and severity would be of interest, as several studies report that alterations to fluid forces can offset lymphatic function and flow [80-86]. Chemotherapy, specifically doxorubicin, have been reported to disrupt lymphatic flow, increasing the risk of LE by four- to five-fold [87-90]. Comparison of cytokine levels in patients who did or did not receive doxorubicin, could be explored.
Numerous studies show that BCRL patients who undergo LVB/VLNT are less prone to cellulitis. Obtaining cellulitis history for all subjects would allow a future study analyzing inflammatory cytokine correlation with cellulitis episode prevalence. Lymphatic function and cytokine levels could be further analyzed in a future study investigating the effects of cytokine levels on lymphatic pumping using NIRF-LI imaging, as a previous study indicated that inflammatory cytokines inhibit lymphatic pumping in mice [36].

3.5 Conclusion

Metric scores obtained from NIRF-LI imaging and plasma cytokine/chemokine MILLIPLEX assay results indicate microsurgeries to be ineffective, at early time points after microsurgeries, in improving lymphatic function in BCRL patients. Our work support previous findings that BCRL is a systemic disease and requires further study. Rather than using perometric arm volume measurement to assess the effectiveness of LE treatment, ICG lymphography (NIRF-LI imaging) is a better tool, as it depicts real-time, “under the skin” lymphatic anatomy and function. Since LE development is a result of lymphatic malfunction, future studies should aim to discover systemic drivers of the disease, thereby leading to treatments for improvement in lymphatic function.


   https://doi.org/10.1113/jphysiol.2004.066118


   https://doi.org/10.1055/s-0038-1635117


   https://doi.org/10.1055/s-0038-1635117


years after breast cancer treatment. *NPJ breast cancer*, 7(1), 70.

https://doi.org/10.1038/s41523-021-00276-y


84. Huabing Li, Huajian Wei, Timothy P Padera, James W Baish, Lance L Munn, Computational simulations of the effects of gravity on lymphatic transport, PNAS Nexus, Volume 1, Issue 5, November 2022, pgac237.


Anna R. Vang is the daughter of Christy Her and Nue Xiong. She graduated from Lakeville North High School in 2016 and went onto obtain a Bachelor of Science in Biology at Augsburg University in 2020. She continued on her educational journey the following year, pursuing a Master of Science degree in Biomedical Sciences with affiliation to the Immunology Program at the University of Texas MD Anderson Cancer Center UTHealth Houston Graduate School of Biomedical Sciences, graduating in 2023.

Linkedin: [www.linkedin.com/in/anna-vang-b8b773157](http://www.linkedin.com/in/anna-vang-b8b773157)